

## Review Article

# Microarray Analyses of Reptiles and Amphibians: Applications in Ecology and Evolution

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### ABSTRACT

**Nicole P. Freire, Michele R. Tennant and Michael M. Miyamoto (2003)** Microarray analyses of reptiles and amphibians: applications in ecology and evolution. *Zoological Studies* 42(3): 391-404. This review examines the potential for microarray analyses of reptiles and amphibians to address ecological and evolutionary questions. Microarray techniques extend the basic principles of nucleic acid hybridization to genome-wide comparisons of gene expression among different cells, tissues, individuals, and/or groups. Reptiles and amphibians are recommended for such global analyses because of their frequent dramatic differences in important biological traits among close relatives (e.g., in reproduction and development), their intermediate positions within the vertebrate phylogeny, and their often-strong responses to environmental stimuli. The potential utility of microarray analyses in ecology and evolution is highlighted using a proposed test of viviparity in the common European lizard and with recent publications on the ecological and evolutionary genetics of other animal systems. <http://www.sinica.edu.tw/zool/zoolstud/42.3/391.pdf>

**Key words:** Microarray, Reptiles, Amphibians, Ecology, Evolution.

### FUNCTIONAL GENOMICS IN THE POST-GENOME ERA

The sequencing of the approximately 3-billion nucleotide base pairs that comprise the human genome is one of humankind's greatest scientific accomplishments. Public and private research groups concurrently unveiled their working drafts

of the human genome in the Feb. 2001 issues of *Nature* and *Science*, respectively (International Human Genome Sequencing Consortium 2001, Venter et al. 2001). The human genome is a relatively recent addition to an ever-growing list of sequenced genomes that include *Saccharomyces cerevisiae* (yeast, Saccharomycetaceae; Goffeau et al. 1997), *Caenorhabditis elegans* (nematode,

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Rhabditidae; *C. elegans* Sequencing Consortium 1998), *Drosophila melanogaster* (fruit fly, Drosophilidae; Adams et al. 2000), *Ciona intestinalis* (sea squirt, Cionidae; Dehal et al. 2002), *Mus musculus* (mouse, Muridae; Chinwalla et al. 2002), *Arabidopsis thaliana* (thale cress, Brassicaceae; *Arabidopsis* Genome Initiative 2000), *Oryza sativa* (rice, Poaceae; Goff et al. 2002, Yu et al. 2002), and over 100 species of other unicellular eukaryotes, bacteria, and archaeans (Bernal et al. 2001). Although such achievements constitute major breakthroughs, the field of genomics acknowledges that the "instruction booklet" of an organism (i.e., its raw sequence) means little unless one knows how to interpret it. Sequencing the entire genome of a species is only the beginning; elucidating how that sequence and its proteins interact to produce a functioning organism is an entirely different and much more complicated task (Lockhart and Winzeler 2000). The completion of a working draft of the human genome signifies a transition to a post-genomics era, in which the focus has shifted from the actual determination of a sequence to its functional analysis.

A major emphasis in post-genomics biology will be to identify and characterize the functions of the multitude of sequenced genes (Vukmirovic and Tilghman 2000). There are many levels at which these questions of gene function can be addressed. Traditionally, RNA-specifying and protein-coding genes have been studied in isolation. While a considerable amount of valuable information has and will continue to be gained through this approach, genomics allows the scope and scale of these studies to be greatly expanded. Genes do not act in isolation but are involved in complex genetic networks and pathways in their determination of biological traits (White 2001). These traits can also be strongly influenced by the environment (Schlichting and Pigliucci 1998). Examination of the effects that entire sets of genes and the environment have on an organism is the basis for the new and growing field of functional genomics.

Functional genomics can be defined as any large-scale, systematic investigation to determine gene function (Brent 2000, Gibson and Muse 2002). Until recently, functional studies of genes were often limited to how genes function and interact at the biochemical and cellular levels. However, due in large part to continuing technological advances, an ever-increasing number of these studies are now focusing on the functional roles and interactions of genes at the physiological, population, and species levels (Fig. 1A). This

broader perspective is rapidly becoming the new research paradigm in biology.

Microarray technology is a relatively new laboratory tool that continues to grow in importance in the field of functional genomics (Fields et al. 1999, Gibson and Muse 2002). Microarrays are miniature chips (with an area of ~1 to 2 cm<sup>2</sup>) that are composed of a solid substrate (often, a glass microscope slide). Onto this solid substrate, specific DNA sequences are immobilized to act as "probes" for their labeled complementary strands (Eisen and Brown 1999). These microarrays are basically extensions of assays such as Southern and Northern blots that use the hybridization property and complementarity of single-stranded DNA to identify specific sequences (Southern 1975, Alwine et al. 1977). Whereas Southern and Northern blots are useful for studies of 1 or a few genes, microarrays can simultaneously quantify the expression of thousands of genes. For example, all of the ~ 6200 genes of yeast have been arrayed onto 1 chip ([http://brownlab.stanford.edu/y\\_array](http://brownlab.stanford.edu/y_array)). Having the entire yeast genome on a single chip allows for studies of how specific environmental changes affect global gene expression (Gasch et al. 2000). With the human genome project nearing completion and with the recent development of even-larger genome chips (i.e., for *D. melanogaster*, *C. elegans*, and *A. thaliana*; <http://www.affymetrix.com/products/arrays/index.affx>), it should be possible in the near future to perform analogous global expression analyses of all ~ 30 000 human genes. The technology is particularly valuable for searching for gene expression differences among different cells, tissues, and individuals (see the prostate cancer example in the next section).

A comparative approach is widely recognized as an integral part of genomic studies (Crawford 2001, Lynch 2001, O'Brien et al. 2001). Species such as yeast, nematode, fruit fly, and mouse continue to serve as model organisms that remain critical for the interpretation and understanding of the wealth of information generated by the Human Genome Project (Clark 1999, Rubin 2001). Presently, little genomic information exists for phylogenetic groups that bridge the relatively simple bacterial, archaean, and unicellular/invertebrate eukaryotic models (e.g., yeast, nematode, and fruit fly) with the more complex mammalian ones, such as the mouse (Fig. 1B). This paucity of genomic information is particularly acute for non-mammalian vertebrates. The recently reported draft of the genome sequence for pufferfish (*Takifugu*

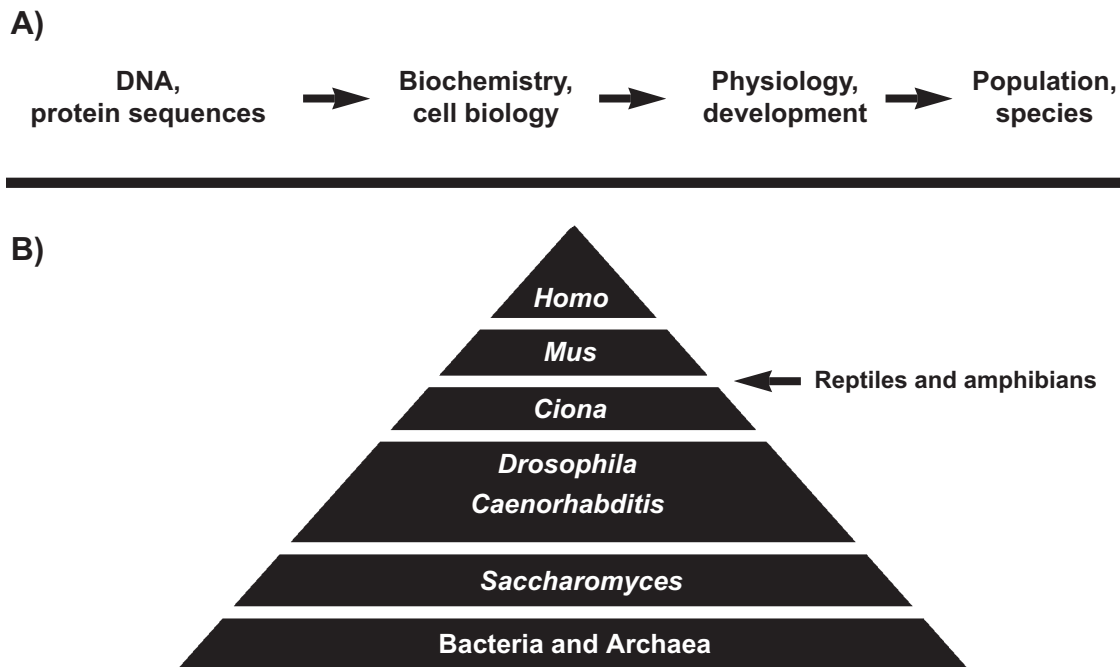
*rubripes*, Tetraodontidae) and the nearly completed one for zebrafish (*Danio rerio*, Cyprinidae) represent the first attempts to fill in these phylogenetic gaps within the Vertebrata (Elgar et al. 1999, Sprague et al. 2001, <http://fugu.hgmp.mrc.ac.uk>). Similarly, as reptiles and amphibians occupy intermediate phylogenetic positions between fishes and mammals, representative members from these 2 classes will also make important contributions to filling in these gaps.

Close relatives of certain reptilian and amphibian species may vary in key biological traits that are usually constant within and among other tetrapod classes (Carroll 1997, Cloudsley-Thompson 1999) (Table 1). These biological differences among closely related forms provide comparative geneticists (including genome scientists) with a relatively common genetic and biological background within which to focus on the underlying factors that shape a particular trait of interest.

These differences may be environmentally, rather than genetically, determined (as is the case of temperature-dependent sex determination in many reptiles). In such cases, the often large physiological and developmental responses of reptiles and amphibians to environmental stimuli make them excellent model organisms for identifying key pathways and events that underlie these more immediate changes (Secor and Diamond 1998).

The purpose of this article is to introduce microarrays and to discuss how this technology may be applied to ecological and evolutionary studies of traits within animal groups (Boake et al. 2002, Gibson 2002, Stearns and Magwene 2003). The possible clinical and medical applications of microarrays have been well documented, but their potential uses in ecological and evolutionary investigations are less well studied. Nevertheless, the potential clearly exists for their extension to other questions focusing on organisms such as reptiles

## Functional Genomics in the Post-Genomics Era



**Fig. 1.** Functional and comparative genomic studies in this century of post-genomics biology. (A) Functional genomics will be a major focus of biology in this century (Lockhart and Winzeler 2000). Such studies of genes and proteins will continue to expand from their functional roles and interactions at the biochemical and cellular levels to those among individuals, populations, and species as genome projects are completed and the technology of genome science continues to advance. (B) Pyramid highlighting the dependence of the human genome project on similar studies of other organisms ranging from simple to complex. This pyramid emphasizes the recognized importance of the comparative approach to a greater understanding of the structure and function of the human genome (Clark 1999). As indicated by the arrow, reptiles and amphibians occupy an important place in this pyramid that connects the simpler model organisms with the more-complex mammalian ones. As such, these 2 groups hold considerable promise as model organisms in future genome studies, as they currently do in many biological areas (Tinsley and Kobel 1996, Pough et al. 2001).

and amphibians (herein referred to as herpetofauna). Historically, amphibians such as *Ambystoma* (mole salamanders, Ambystomatidae; Shaffer 1993), *Rana* (true frogs, Ranidae; Hillis 1988), and *Xenopus* (clawed frogs, Pipidae; Tinsley and Kobel 1996, Altmann et al. 2000, Beck and Slack 2001) have been the principal herpetofaunal organisms used as model systems. This paper highlights the potential for other herpetofaunal elements, especially reptiles, to serve as model organisms in the post-sequencing era of functional genomics.

### GENE EXPRESSION PROFILING AND MICROARRAYS

Gene expression profiling is a type of functional genomic analysis in which the activities of thousands to tens of thousands of genes are monitored by analyzing and quantifying the mRNA transcripts of specific cells and tissues at particular times and under distinct conditions (DeRisi et al. 1997, Lockhart and Winzeler 2000). There are a number of laboratory methods that have been developed over the last decade for this type of high-throughput gene expression analysis, including cDNA sequencing (Okubo et al. 1992), mRNA differential display (Liang et al. 1993), serial analysis of gene expression (SAGE; Velculescu et al. 1995), and microarray analysis (Schena et al. 1995). While all of these methods provide information on the type and/or abundance of expressed genes, microarray investigations are the only ones that allow for comprehensive and reliable parallel analysis of gene expression among different cells,

tissues, and individuals (Duggan et al. 1999). Due to both their greater flexibility and reliability, microarrays are currently being promoted as the method of choice for high-throughput analyses of gene expression (Clark 1999, Gibson and Muse 2002). For further reviews on microarrays, see Brown and Botstein (1999), Lockhart and Winzeler (2000), and Holloway et al. (2002).

There are 2 major types of arrays: the complementary DNA microarray (cDNA array) and the oligonucleotide microarray (oligo array) (Duggan et al. 1999, Lipshutz et al. 1999, Gibson and Muse 2002). Both rely on the same basic hybridization principle outlined above and can be used effectively in parallel gene expression studies. Nevertheless, they differ in many fundamental details (Lockhart and Winzeler 2000). For example, oligonucleotide arrays are synthesized using sequence information, whereas cDNA arrays can be synthesized with or without prior sequence knowledge (Lipshutz et al. 1999). In this paper, we focus on cDNA microarrays since genomic sequences are unavailable for most herpetofaunal species, a situation that is unlikely to change in the immediate future.

To illustrate the basic steps in a standard cDNA microarray study, in which sequence information is not used, we present a modified and simplified protocol that combines the procedures of Eisen and Brown (1999), Livesey et al. (2000), and Xu et al. (2000) (Figs. 2, 3). To better demonstrate how and why specific comparisons of gene expression are made, we present this protocol in terms of the microarray study of samples of prostate cancer (PC) versus a normal prostate

**Table 1.** Some reptilian and amphibian examples that illustrate important variation in major biological features of reproduction and development (Duellman and Trueb 1986, Pough et al. 2001). In many cases, these traits vary substantially within a species or among closely related taxa, thereby providing a recent phylogenetic context to examine the problem using functional genomic analysis

Major reproductive/developmental attribute	Representative groups
Parthenogenesis, gynogenesis, and hybridogenesis	Various lizards, <i>Rana</i> , and <i>Ambystoma</i>
Limb reduction or loss	Snakes, various lizards, and salamanders
Direct development in amphibians	Various caecilians, frogs, and salamanders
Viviparity	Many squamates (e.g., <i>Lacerta vivipara</i> ) and some amphibians
Environmental (temperature-dependent) sex determination	Crocodylians, <i>Sphenodon</i> , most turtles, and some lizards
Neoteny and paedomorphosis	Many salamanders

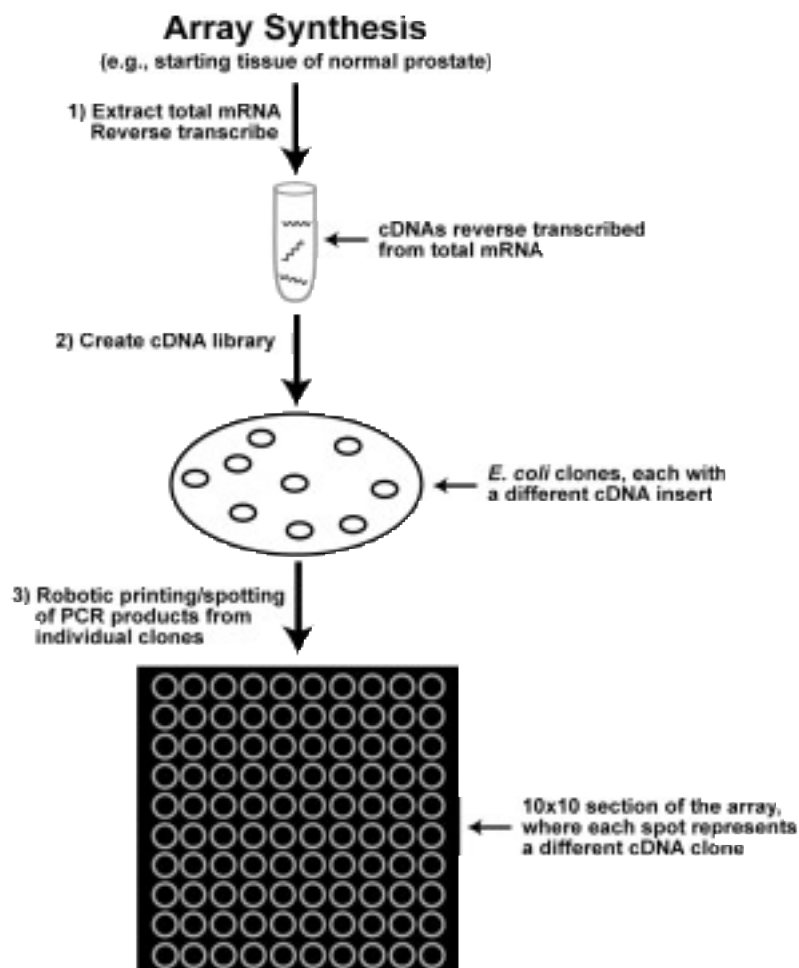
(NP) by Xu et al. (2000).

The cDNA microarray is first constructed from the cDNA library of the tissue of interest (Fig. 2). cDNA is DNA that is complementary to transcribed mRNA and thus represents a gene that is expressed within the tissue. A cDNA library is then a collection of all expressed genes of tissues and cells from which the mRNA was extracted. For comparison of the gene expression of NP and PC, a cDNA library can be constructed from NP tissue by isolating its total mRNA population, reverse-transcribing the mRNA into cDNA with the enzyme reverse transcriptase, and then transforming the *Escherichia coli* host with plasmids that carry the cDNAs as inserts. The inserts of different clones of the library are then amplified by the polymerase chain reaction (PCR, with vector-spe-

cific primers), and their products are fixed onto an array. In this fashion, thousands of genes are fixed onto a single microarray for the purpose of comparing the gene expression profiles of NP and PC under different experimental treatments and conditions.

One drawback to synthesizing the array from clones derived from cDNA libraries is that many of the spots on a chip will be redundant. This is because certain genes are expressed more often than others in any given tissue and as such these genes have more spots represented on the array. To minimize this redundancy, clones of a cDNA library can be normalized with techniques such as subtractive and colony hybridization (see Xu et al. 2000) before spotting them onto the array.

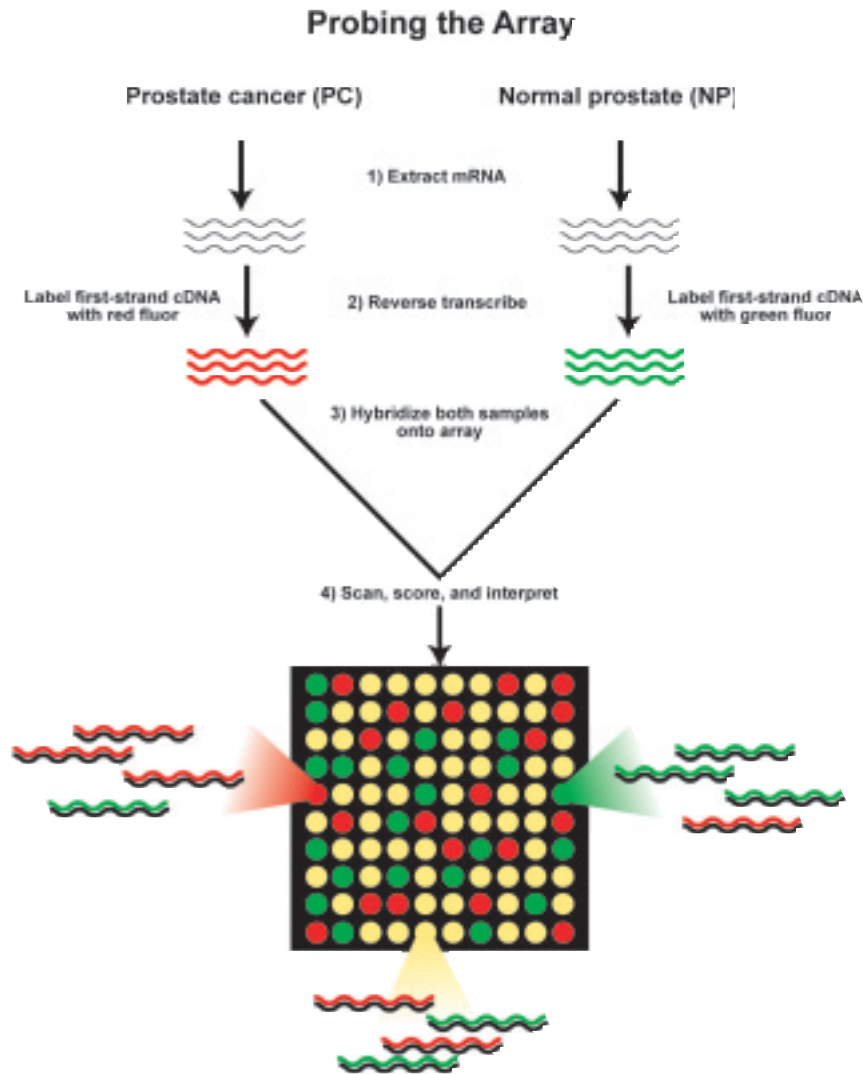
Once synthesized, the next step is to probe



**Fig. 2.** Basic scheme for the synthesis of a cDNA microarray, as modified from Eisen and Brown (1999), Livesey et al. (2000), and Xu et al. (2000). (1) The total mRNA pool is extracted from the tissue of interest and reverse-transcribed into total cDNA. (2) Each cDNA is then amplified through cloning to create a cDNA library. (3) A specific amount of cDNA from each clone (as PCR products) is fixed onto unique positions of the array.

the array by hybridizing it to the samples of interest. To do this, total mRNA from both the PC and NP samples are isolated and separately reverse-transcribed into first strand cDNA (Fig. 3). During reverse transcription, these cDNAs can be fluorescently labeled with dyes of different colors, such as red for cancer and green for normal. These 2

pools of fluorescently tagged (single-stranded) cDNAs are then competitively hybridized to the same NP array. The pattern of fluorescence is then scanned and analyzed computationally. Genes on the array that are differentially expressed between the 2 samples produce distinct patterns of fluorescence. In cases where genes in



**Fig. 3.** Basic scheme for probing a cDNA microarray, as modified from Eisen and Brown (1999), Livesey et al. (2000), and Xu et al. (2000). The normal prostate (NP) microarray in this diagram was generated as shown in figure 2. (1) Total mRNA is extracted from prostate cancer (PC) and NP. (2) Both are reverse-transcribed into 1st-strand cDNAs that are fluorescently labeled red or green. (3) The 2 pools of labeled cDNAs are then competitively hybridized to the NP array. (4) The fluorescence of each spot is analyzed and interpreted. Spots on the array that are red represent genes that are over-expressed in PC relative to NP. In contrast, relatively green spots represent genes that are under-expressed in PC versus NP. Yellow spots represent genes that are similarly expressed in both tissues. In this hypothetical example, every spot hybridizes to at least the cDNA of NP, because this microarray was derived from mRNA of this tissue (Fig. 2). However, many studies rely instead on microarrays that are more general and broader in terms of their represented genes (e.g., Gasch et al. 2000). In those cases, certain spots on the microarray will remain black after probing, because the labeled cDNAs of neither source will hybridize to them. These black spots signify genes that are not expressed in either test sample.

PC are over-expressed relative to those in NP, spots on the array would show a higher intensity of red fluorescence. Conversely, where genes in PC are under-expressed relative to those in NP, these spots would exhibit a higher intensity of green fluorescence. In this fashion, the pattern of fluorescence identifies those genes that are over- or under-expressed in a tissue as compared to another, as well as those that are expressed equivalently, such as those producing yellow spots on the microarray. By use of such a cDNA microarray, genes with different expression patterns can be detected and then targeted for further follow-up studies to confirm differences in their expressions and to identify and characterize them (Chuaqui et al. 2002, Slonim 2002).

#### **APPLICATIONS OF MICROARRAYS IN ECOLOGY AND EVOLUTION: THE EXAMPLE OF VIVIPARITY IN *LACERTA VIVIPARA***

Feder (1999) reviewed 5 major approaches for identifying the key genes that are responsible for a phenotypic trait or difference: 1) the gene-to-phenotype approach; 2) the phenotype-to-gene approach; 3) the genomics approach; 4) the random mutagenesis approach; and 5) the candidate gene approach. In this review, the emphasis is on microarrays as a tool for identifying candidate genes that most likely underlie a particular phenotypic difference. Thus, this review emphasizes their utility in candidate gene discovery following a top-down, phenotype-to-genotype approach (i.e., category 2; see also Boake et al. 2002).

Approaches 1 to 5 are complementary, with the phenotype-to-genotype focus of this review naturally leading to category 5. That is, follow-up experiments are normally done with candidate genes from a microarray study to confirm their expression differences, to establish their identities, and to better understand their possible roles in determining a trait. These follow-up experiments frequently include DNA sequencing of the candidate genes, bioinformatics analyses of their sequence relationships, structure, and function, Northern blotting, quantitative PCR, tissue in situ hybridization, and immunohistochemistry (Chuaqui et al. 2002, Slonim 2002). Indeed, at least in certain groups (e.g., *Xenopus* and *Drosophila*), the most promising candidates from these follow-up tests may then be directly evaluated for the phenotypic effects of their structural and regulatory changes in transgenic animals (Sparrow et al.

2000, Tatar 2000, Hirsch et al. 2002). Such further testing of candidate genes represents an application of the gene-to-phenotype approach (category 1). In this light, microarrays can be seen as a new tool that spans several approaches for identifying those key genes that underlie complex biological traits.

Microarray analyses hold considerable promise for elucidating the pathways and events that underlie many traits, not only those within a biomedical context but also those with ecological and/or evolutionary significance (Boake et al. 2002, Gibson 2002, Stearns and Magwene 2003). This especially applies to cases where a complex trait varies extensively or dramatically within a species or among closely related taxa (Table 1). A prime example is the reproductive mode within reptiles. Although most vertebrates are oviparous (egg-laying), viviparity (live-bearing) is found in a wide variety of vertebrate classes (i.e., it characterizes most mammals, as well as some fishes, amphibians, and reptiles) (Blackburn 1999a). The fact that viviparity has convergently evolved in different groups suggests some common selective benefits to this reproductive mode across different life histories and habitats (Wake 1992, Andrews and Mathies 2000). Squamate reptiles in particular represent excellent systems within which to study the evolution of viviparity. Within this group, viviparity has evolved independently ~100 times, relatively recently, and at low taxonomic levels (Shine 1985, Blackburn 1999b 2000).

Viviparity can be defined as the retention of an embryo within the uterus until development is complete (Guillette 1993). A number of factors have been established as important in the evolution of viviparity: 1) an increase in the retention time of the egg within the uterus; 2) a reduction or loss of the eggshell; 3) increased nutrient transfer to the embryo; 4) the development of a placenta; and 5) a reduction in yolk protein (Packard et al. 1977). Guillette (1993) developed a physiological model of how viviparity may have evolved through the loss of eggshell glands (and correspondingly, thinning of the eggshell as in factor 2). This hypothesis argues that a thin eggshell permits increased diffusion of embryonic factors to the uterus, thereby leading to retention of the corpus luteum. The corpus luteum is the short-lived endocrine organ of the ovaries that forms after ovulation and that primarily secretes the hormone progesterone for egg/embryo retention and the maintenance of pregnancy (Xavier 1987, Callard et al. 1992).

Species with both modes of reproduction (i.e., those with both viviparous and oviparous populations) are ideal models for studying the evolution of viviparity (Guillette 1993, Fairbairn et al. 1998). One such species that has been extensively studied is *Lacerta vivipara* (common European lizard, Lacertidae). This species includes isolated oviparous populations in southwestern France, northern Spain, and Slovenia and viviparous ones throughout the rest of its largely European distribution (Surget-Groba et al. 2001). These populations are widely accepted as conspecific, even though they conform to separate evolutionary lineages (Bea et al. 1990, Guillaume et al. 1997, Heulin et al. 1999). Their recognition as a single species is based on their small genetic (allozyme and mitochondrial 16S ribosomal RNA) distances, their extensive morphological and ecological similarities, and their ability to hybridize (see below). Further phylogenetic analyses of these populations indicated that viviparity probably evolved only once and relatively recently within this species (Surget-Groba et al. 2001).

In the laboratory, viable and fertile  $F_1$  hybrids can be produced between oviparous and viviparous lineages of *L. vivipara* (Arrayago et al. 1996). The viviparous and oviparous forms are distinguished by their eggshell thickness and composition (a mean of 9  $\mu$  and no calcification versus a mean of 40  $\mu$  and complete calcification, respectively) and stage of embryonic development at parturition or egg-laying (stage 40 or complete development versus stages 31 to 34, respectively).  $F_1$  hybrids are oviparous, but show reproductive traits that are intermediate between their parents (a mean eggshell thickness of 21  $\mu$ , incomplete calcification, and eggs oviposited at stage 35 or 36). These results are consistent with the physiological model in that a reduced thickness of eggshell is associated with increased egg retention and embryonic development at oviposition (Guillette 1993). However, a more-direct test of the physiological model would be to determine whether the viviparous form has fewer eggshell glands than the oviparous one. This prediction is founded on the widespread association of fewer eggshell glands, in viviparous forms, compared to the oviparous counterparts, of most lizard groups with both reproductive modes (Christiansen 1973, Guillette et al. 1989, Palmer et al. 1993).

#### A MICROARRAY TEST OF THE PHYSIOLOGICAL MODEL OF VIVIPARITY

Guillette (1993) proposed several mechanisms to explain the reduction in the numbers of eggshell glands in viviparous populations relative to their oviparous counterparts. These mechanisms included gene regulation, both during and after transcription, as well as *cis*- and *trans*-acting factors for activating eggshell gland formation. One possible mechanism involved a reduction in the number of protein receptors for the hormone estrogen. Estrogen is known to influence the formation of eggshell glands in reptiles (Blackburn 2000), and like other steroid hormones, it exerts its effects by binding to receptor proteins (estrogen receptors; ERs) (Grandien et al. 1997). This binding activates the ERs such that they attach to conserved estrogen response elements (EREs) in the DNA. These EREs serve as *cis*-enhancer sequences that result in the transcriptional activation of their associated structural (protein-coding) genes, when complexed with activated ERs. Along these lines, Guillette (1993) suggested that a reduction in the number of ERs for eggshell gland formation may have led to fewer eggshell glands and thus to eggshell thinning in viviparous populations.

As outlined in figure 4, we adopt this proposed mechanism to illustrate how a microarray analysis could be employed to identify the structural and regulatory genes controlling viviparity. Figure 4 presents a hypothetical situation, where 3 structural genes for eggshell gland formation (I, II, and III) are differentially expressed between oviparous and viviparous females. The purpose of this hypothetical example is to illustrate the potential power of microarrays for identifying those genes (I, II, and III and the structural locus for the ER itself) that are most likely responsible for traits of ecological and evolutionary importance. In this hypothetical example, genes I, II, and III are fully expressed in oviparous females, thereby leading to the development of many eggshell glands in their uteri and a thick calcified shell around their embryos. Conversely, this example suggests that such activation is reduced in viviparous females due to lower levels of gene expression for ERs. Consequently, fewer activated complexes of ERs become available for transcriptional enhancement of I, II, and III. In turn, fewer eggshell glands and secretory materials form in the uteri of viviparous females, thereby leading to a reduction in thickness of their eggshells. As a result, these thinner eggshells would allow for greater molecular signaling between the mother and her offspring, which would produce longer egg and embryo retention



times.

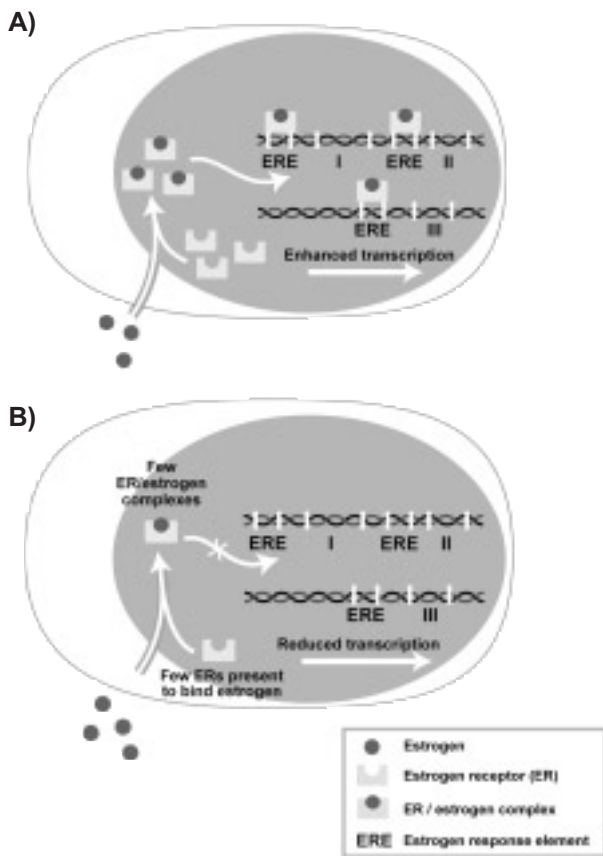
Very little sequence information, particularly for nuclear genes, is currently available for *L. vivipara* or for its genus and family in genetic databases (e.g., GenBank; Benson et al. 2003). Given this paucity of sequence information, a cDNA microarray approach could be used to test the genetic basis of viviparity and its evolutionary origin in this particular case. Thus, the basic methods for building and probing a cDNA microarray, as presented for samples of normal versus cancerous prostates (Figs. 2, 3), could be adapted to screen the genomes of *L. vivipara* for those genes that are differentially expressed between oviparous

and viviparous females. In this case, the microarray would be built from cDNA libraries of the uterine lining during the initial development of the eggshell glands (Blackburn 1998). Using this tissue is essential to maximize the odds of representing those genes for the reproductive mode on the cDNA microarray.

As shown in figure 5, this cDNA microarray approach should identify the ER gene, as well as the 3 hypothetical genes for eggshell gland formation. In this case, the genes should display elevated expression in oviparous compared to viviparous females. Similar increases in expression could be detected with hierarchical clustering and multivariate methods for pattern discovery and class prediction, followed by further study in isolated or combined analyses (Chuaqui et al. 2002, Slonim 2002). In particular, DNA sequencing and subsequent sequence comparisons of genes I, II, and III could be used to verify that they share common ERE motifs that are diagnostic for genes under the control of estrogen (Grandien et al. 1997). Thereafter, one could consider the use of transgenic animals with different regulatory patterns to directly test for effects in vivo of reduced estrogen activation on gene expression (Sparrow et al. 2000, Tatar 2000, Hirsch et al. 2002). For example, available technology is reaching a point where one can consider inserting additional copies of the ER gene into fertilized eggs and early embryos of the viviparous form. In this case, one would predict that these extra copies of ERs would enhance the response of these transgenic females to estrogen, thereby leading to greater development of their uterine eggshell glands and thicker eggshells.

Not all viviparous squamates studied to date follow the pattern of eggshell reduction that led Guillette (1993) to emphasize eggshell thickness in his physiological model. In a comparison of oviparous sceloporines (fence lizards, Phrynosomatidae), Mathies and Andrews (2000) found no association between eggshell thickness and either the extent of egg retention or embryo development within the mother. Given this lack of an association between eggshell thickness and “viviparity,” these authors argued for 2 separate pathways in the transition from egg laying to live bearing. In the 1st pathway, eggshell thinning is directly tied to viviparity (as in *L. vivipara*), whereas in the 2nd, it evolves secondarily (as in sceloporines).

Once again, by adopting the general approach of this study (Figs. 4, 5), microarray experiments could be designed to test whether the underlying genetic mechanisms of viviparity in *L.*



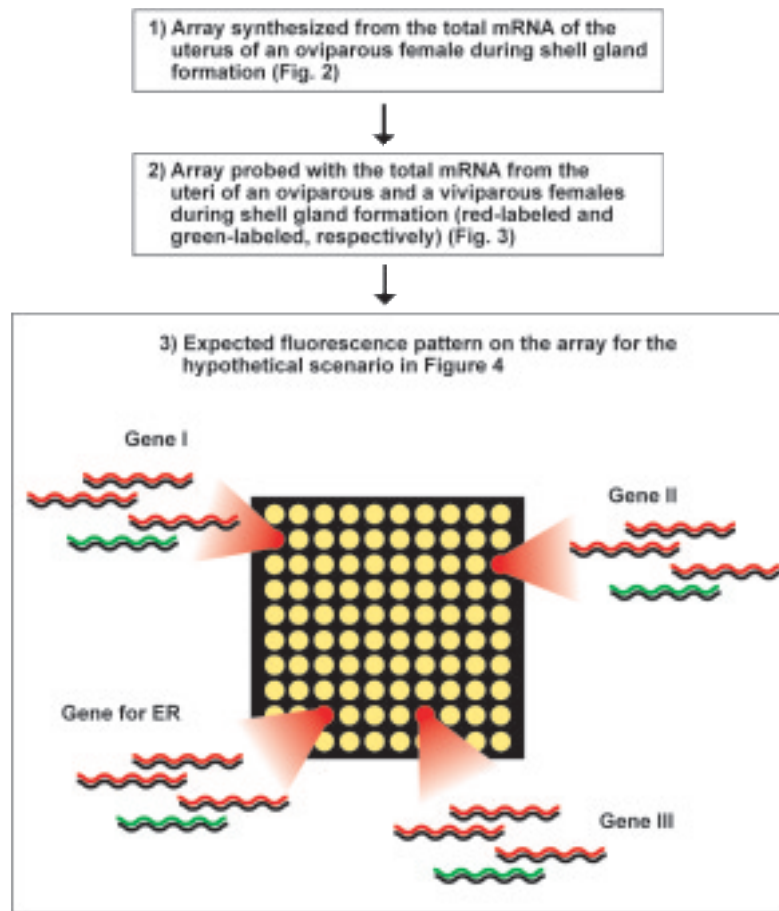
**Fig. 4.** Hypothetical example of 3 structural genes for shell gland formation (I, II, and III) which are under the control of estrogen and its estrogen receptor (ER). (A) This hypothetical diagram illustrates the up-regulation of I, II, and III and the ER gene in an oviparous lizard. In this case, estrogen binds to its receptor protein to generate activated ER/estrogen complexes that attach to the estrogen response elements (EREs), thereby enhancing the transcription of their associated structural genes. (B) In a viviparous lizard, 1 possibility is that the ER gene is down-regulated, thereby reducing the formation of ER/estrogen complexes. Thus, there is little transcriptional enhancement of genes I, II, and III.

*vivipara* are the same or different in sceloporines. These tests could be expanded to include the 2 other well-documented species with both oviparous and viviparous populations (*Lerista bouganvillii* and *Saiphos equalis* (southern Australian skinks, Scincidae)). Like in *L. vivipara*, viviparity in *L. bouganvillii* is associated with eggshell thinning (Qualls 1996). In contrast, as in some sceloporines, certain populations of *S. equalis* produce eggs with relatively thick eggshells, which hatch only days after ovipositing (Smith and Shine 1997). By expanding these comparisons even further to include representative tropical groups with both reproductive modes, these microarray experiments could lead to new insights into the general ecological and evolutionary factors and numbers of different pathways that underlie the ~100 independent origins of viviparity

within squamates (Shine 1985, Blackburn 1999b).

## FUTURE PROSPECTS

While our review was being completed, a number of microarray studies were published for the model amphibian, *Xenopus laevis*. In all of these recent publications (except for one), microarrays were employed to monitor gene expression during the ontogeny of this species under both natural conditions and in the presence of an environmental pollutant (Altmann et al. 2001, Crump et al. 2002, Munoz-Sanjuán et al. 2002, Veldhoen et al. 2002). In the 1 exception, this technology allowed for testing the hybridization efficiencies of different antisense oligonucleotides to their target RNAs from *X. laevis* (Sohail et al. 2001). At this time,



**Fig. 5.** Outcome of a hypothetical microarray analysis between oviparous and viviparous females of *Lacerta vivipara* following the scenario in figure 4. (1) The array is synthesized from oviparous mRNA as in figure 2. (2) The array is then probed with the total mRNAs from the uteri of oviparous and viviparous females following figure 3 (red-labeled and green-labeled, respectively). (3) Expected fluorescence pattern for the hypothetical scenario in figure 4. The greater expressions of the ER, I, II, and III genes are revealed for the oviparous as compared to the viviparous female.

these recent analyses of *X. laevis* represent the only published applications of microarrays to a herpetofaunal species.

As reflected by Crump et al.'s (2002) analysis of an environmental pollutant, a relatively large number of microarray studies with ecological and evolutionary significance were also published for different animal groups during completion of our review. Among the 1st of these ecological and evolutionary studies was that by Gracey et al. (2001), who used microarrays to test gene expression responses of *Gillichthys mirabilis* (long-jaw mudsucker, Gobiidae) to hypoxia, which is an important physiological factor of general interest to ecologists and evolutionary biologists as well as biomedical researchers (Woakes et al. 1991). In the process, their study provided the 1st "proof of principle" that the microarray technology can be applied to animal species with little or no available genomic information (Pennisi 2002). Since then, the number of these published microarray studies has continued to grow, thereby documenting the utility of this technology to ecology and evolution (e.g., Daborn et al. 2002, Enard et al. 2002, Konu and Li 2002, Oleksiak et al. 2002, Pletcher et al. 2002, Robinson 2002, Wayne and McIntyre 2002, Rifkin et al. 2003).

Although best known for their applications in gene expression studies, microarrays are also becoming important as a tool for genotyping individuals within and among populations and for comparing sequence variations among different regions of the genome (Frazer et al. 2001, Fan et al. 2002, Knudsen 2002). The cDNA microarray approach remains the primary method for global analyses of gene expression in species with little or no sequence information (Gibson 2002). Nevertheless, this should not obscure the obvious fact that genomic sequence information is always desirable, particularly when accompanied by functional annotations. Such sequence information can allow for the design of custom oligonucleotide arrays that are aimed at particular traits or biological questions. Indeed, given such information, researchers can now order custom oligonucleotide arrays for their study species from biotechnology companies (see <http://www.affymetrix.com/products/arrays/specific/custom.affx> and <http://www.chem.agilent.com/scripts/pds.asp?1Page=2989>, as well as Pennisi 2002 for a comment on collaborating with certain research centers on the design, construction, and application of cDNA microarrays).

As an introduction to microarray technology, this review has focused on general aspects of data

acquisition and gene expression profiling, rather than on details of experimental design, statistical analysis, and interpretation. Nevertheless, this emphasis should not obscure the fact that these statistical and bioinformatics aspects of microarray studies constitute an active research area of major significance (Knudsen 2002). This sub-discipline is concerned with the normalization and transformation of microarray data, with the statistical testing of gene expression differences, and with the interpretation of results in terms of regulatory and biological networks and pathways (Quackenbush 2002, Slonim 2002). In particular, the statistical testing of gene expression differences covers both reference sample and analysis of variance (ANOVA) approaches, whereas the interpretation of results includes clustering and multivariate methods for pattern discovery and class prediction (Gibson and Muse 2002). These analytical concerns are naturally interconnected with those associated with sampling, replication, and other aspects of experimental design (Churchill 2002). In short, microarray data are very extensive and complex, and newer methods of analysis and interpretation are constantly being developed. Given this complexity and changing landscape, collaborating with statisticians is strongly recommended.

Microarrays are among the 1st of many technologies that promise to revolutionize the life sciences in this new century of post-genome biology (van Berkum and Holstege 2001, Duyk 2002, Gibson and Muse 2002). The microarray approach, as outlined herein, can be used for transcriptional profiling at the RNA level, where much of gene regulation is thought to occur (Lewin 2000). However, the emerging field of proteomics is rapidly developing complementary tools for the large-scale study of gene expression at the translational and post-translational levels of protein synthesis (Pandey and Mann 2000, MacBeath 2002). In combination with DNA microarrays, these complementary tools for cell-wide studies of proteins will allow a more-comprehensive understanding of gene regulation through all levels of its expression. As these genomics and proteomics technologies continue to advance and become more readily available, we further encourage ecologists and evolutionary biologists to consider how these powerful new approaches for global gene expression can benefit their studies of organismal adaptation and change (Boake et al. 2002, Gibson 2002, Stearns and Magwene 2003).

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